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PRINCIPAL INVESTIGATOR: Russell J. Vanderboom, Ph.D.

CONTRACTING ORGANIZATION: Mayo Foundation
Rochester, Minnesota 55905

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Vanderboom, Russell J., Ph.D.

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In order to study the effect of the mucin Muc1 on tumor growth in the mammary glands of mice, a chemical carcinogenic combination of medroxyprogesteone acetate (MPA) and nitroso-methylurea was administered to wildtype C57BL/6 mice and mutant knock-out C57BL/6 mice unable to express Muc1. One week after implantation of time release MPA pellets, mice were injected with NMU. Mice received monthly injections of NMU for three months. Treatment failed to induce more than one tumor. Mice experienced excessive morbidity as a result of the chemical treatment. The trial was aborted after six months. Chemical carcinogenesis using MPA/NMU was not an efficient means to induce tumors in C57BL/6 mice for the study of Muc1 effect on growth immune response, or cell-matrix interactions in mice.

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Russell J. Vandenberg 8/21/98

PI - Signature Date

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Introduction

This report summarizes progress and pitfalls encountered during the first year of funding from Army Grant Number DAMD17-97-1-7274. These studies addressed the role of a mammary epithelial expressed mucin, Muc1, during growth and development of breast tumors in mice. For clarity, the murine mucin is labeled 'Muc1' and the human mucin is labeled MUC1.

Muc1 is normally expressed as a large transmembrane glycoprotein during development and lactation in mice, as well as in humans and other mammals (reviewed in Gendler, 1995). The human MUC1 gene encodes a protein that is polymorphic and includes variable numbers of tandem repeats (from 30 to 90) consisting of 20 amino acid residues in the extracellular domain (Gendler, 1990; Lan, 1990; Ligtenberg, 1990; Wreschner, 1990). Each repeat sequence contains five serine and threonine residues which serve as potential O-glycosylation sites. Glycosylation constitutes approximately 50% of the glycoprotein in the normal mammary gland, where MUC1 has a mass of 250 to 500 kDa (Shimizu, 1982). The apoprotein ranges from 125k Da to 225 kDa, depending on the individual genetic polymorphism. The molecule consists of an extracellular domain (approximately 1150 to 2200 amino acids, depending on the number of tandem repeats), a single transmembrane domain (28-31 amino acids) and a cytoplasmic tail (69-72 amino acids) that appears to interact with the actin cytoskelaton (Parry, 1990).

Murine Muc1 is the homologue of human MUC1 (Spicer et al., 1991). It is a smaller version of the human protein, with comparable cytoplasmic tail and transmembrane domains but with a reduced, fixed number of random repeats (sixteen). Murine Muc1 retains 87% homology to human MUC1 in the transmembrane and cytoplasmic domains. All exon/intron boundaries of the murine Muc1 genomic structure are conserved with the human gene. The promoter region is 72% homologous between species. Human and murine MUC1 genes show similar expression patterns (Pemberton et al., 1992; Spicer et al., 1991)).

MUC1 expression in cancer cells differs in several ways from the MUC1 characteristic of normal mammary epithelial cells. In cancer, MUC1 is overexpressed, with 10 to 100 times as many molecules as found in normal cells (Zaretsky, 1990). The cancer mucin molecule is aberrantly glycosylated (Braga et al., 1992; Girling et al., 1989; Devine et al., 1990; Taylor-Papadimitriou, 1991). The molecule contains fewer, shorter polysaccharide O-linked carbohydrate side chains (Hull et al., 1989). The molecule is distributed in an non-polarized manner across the cancer cell membrane, which contrasts to the apical luminal localization of MUC1 in normal mammary epithelial cells. Tumor cells shed the glycosylated extracellular domain of MUC1 into plasma; the presence of elevated MUC1 in the serum is an indicative marker of cancer in human breast cancer patients. One in nine women develop breast cancer (Harris, 1992); nine of ten breast cancer patients exhibit elevated MUC1 expression in serum (Burchell et al., 1984; Metzgar et al., 1984; Hayes et al., 1985; Hilkens et al., 1986). The molecule is aberrantly glycosylated in more than 92% of primary and metastatic breast cancers (Burchell, 1987; Zotter, 1988; Girling, 1989).

Muc1's structural and biochemical characteristics suggest a role for this glycoprotein in cell-cell and cell-matrix interactions. Muc1 may be involved in immune recognition or cellular adhesion. The variations in MUC1 expression in cancer cells compared to normal cells suggest important physiological functions. MUC1 has been implicated in altering immune recognition and cell adhesion, thereby affecting cell survival. *In vitro* studies have shown that MUC1 inhibited NK cell and cytotoxic T cell functions (Hayes, 1990; van de Wiel-van Kemenade, 1993). This inhibition may be steric in nature due to the extended extracellular structure of the protein (Jentoft, 1990). Alternatively, the underglycoslyated MUC1 from tumor cells may have discreet crosslinking properties with CTL cells that diminish their effectiveness against those tumor cells (Barnd, 1989).

The presence of large amounts of MUC1 on the non-polarized tumor cell surface may affect adhesion. Ligtenberg (1992) demonstrated that cell lines that expressed high levels of MUC1 did not aggregate as effectively as control cells. Hilkens suggested that steric properties of MUC1, rather than negative charge, interfered with E-cadherin mediated cell-cell interactions (Wesseling, 1996). *In vitro* studies have also demonstrated that MUC1 interfered with cell-matrix interactions mediated by β1 integrins in human breast cell lines (Wesseling, 1995). Alternatively, MUC1 may act as an adhesive molecule by presenting carbohydrates as ligands for selectin-like molecules and thus aid metastatic dissemination (Rice, 1989; Aruffo, 1992). Depending on its variable carbohydrate composition, MUC1 may have affinity for selectin-like molecules (Baekckstrom et al., 1991; Hanski et al., 1993), which implicates it in cell migration and metastasis. MUC1 has been described as containing the sialyl Lewis^X and sialyl Lewis^A carbohydrate structures, which are known ligands for P- and E-selectins (Baeckstrom, 1991; Majuri, 1992; Hanski, 1993). These data support a role for MUC1 involvement in motility. Depending on its variable carbohydrate composition, MUC1 may also act as a ligand for selectin-like molecules (Baekckstrom et al., 1991; Hanski et al., 1993), which implicates it in cell migration and metastasis. While MUC1 can be immunogenic, it can paradoxically function immunosuppressively. MUC1 from carcinomas can be a target for immune responsor cell recognition by cytotoxic T lymphocyte (CTL) cells (Barndt et al., 1989). While that suggests that the immune system could target MUC1 bearing tumor cells, carcinoma cells shed the extracellular domain of MUC1 into the circulation (Burchell et al, 1984; Metzger et all, 1984; Hayes et al, 1985; Hilkens et al., 1986). Thus, free MUC1 in the serum may bind up CTL recognition sites, preventing the immune cells from targeting MUC1 producing tumor cells (van de Wiel-van Kemenade et al., 1993).

To examine the role of Muc1 in tumor development in our laboratory, Spicer et al., (1995) mutated the Muc1 gene by homologous recombination and created mice with no Muc1 protein expression. Remarkably, the mice null for Muc1 expression had no obvious developmental phenotype. Mice reproduced, lactated, and were generally healthy. Only a subtle precocious developmental difference was observed in ductal morphology of mutant Muc1 knockouts (Rowse, personal communications).

When primary breast tumors were induced by polyoma middle T antigen expression in mutant Muc1 mice, the growth rate of tumors was significantly slower than

observed in tumors from non-mutant middle T antigen transgenic wildtype mice (Spicer et al., 1995). We wished to verify this effect on tumor growth by examining another model for mammary gland cancer. We choose the MMTV-driven unactivated neu mice which develop unifocal mammary gland tumors at approximately 7 months of age and exhibit high rates of metastasis. This model is more biologically relevant than the MTag mice that develop very aggressive tumors throughout the entire mammary gland at approximately 60 days of age. However, mouse line strain differences imposed genetic effects upon tumor growth response. Muc1 mutants on the C57Bl/6 background exhibited inherent tumor resistance compared to F1 or FVB Muc1 mutants. In order to circumvent inter-strain genetic variance in tumor growth and development studies, the present grant proposed to initiate tumor development using chemical carcinogenesis. Pazos et al. (1991) demonstrated chemical induction of murine mammary tumors using N-methyl-N-nitrosourea (MNU) and medroxyprogesterone acetate (MPA. Mice treated with both MNU + MPA showed 79% incidence with a latency of 154 ±19 days. Tumors were mammary adenocarcinomas of the B type of Dunn's classification (Pazos, 1991).

My **hypothesis** for these studies was that the high level of expression of MUC1 in tumors facilitates progression of the tumors. The manner by which tumor expression of MUC1 benefits the malignancy may have included: 1) interference with immune response effector cell recognition of tumor cells, or 2) alteration of adhesive properties of the tumor cell membranes. High concentrations of Muc1 expressed on the surface of the tumor cells are thought to protect tumors from immune surveillance by restricting access of lymphocytes to MHC molecules on the surface of the tumor (Codington, 1979; Hayes, 1990; van de Wiel-van Kemenade, 1993) or by rendering the tumor cells resistant to lysis by natural killer cells (Sherblom, 1986; Bharathan, 1990). *In vitro* studies have shown that high levels of expression of MUC1 affected cell aggregation and decreased the adhesiveness of cells to extracellular matrix molecules (Ligtenberg, 1992; Wesseling, 1995; 1996).

Specific Aims

1. I proposed to induce tumors in Muc1-deficient mice and in transgenic and wildtype control mice using chemical carcinogens. I aimed to compare the rate of tumor growth in the Muc1 -/- mutant mice with the control wildtype mouse that expresses the mouse Muc1 protein and with the transgenic mice expressing the human MUC1 protein.

2. In order to assess the potential role of Muc1 in tumor recognition by NK and CTL cells, I proposed to examine the effect of immune modulation on the growth rate of tumors in Muc1-deficient and control mice.

3. I proposed to examine the characteristics of mammary tumor cells derived from the Muc1 deficient, transgenic, and control mice to recognize and bind extracellular matrix ligands including fibronectin, laminin, collagen type iv and v, E-selectin, and hyaluronic acid.

Body of the report

The Statement of Work from this proposal delineated objectives from each specific aim to be completed in the first year of work. These include:

Specific Aim 1: to induce tumors in Muc1-deficient mice and in transgenic and wildtype control mice using chemical carcinogens and to compare the rate of tumor growth in the Muc1 -/- mutant mice with the control wildtype mouse that expresses the mouse Muc1 protein and with the transgenic mice expressing the human MUC1 protein.

Task 1	Months 1-12	Analysis of tumors generated in Muc1 deficient, wildtype, and transgenic mice. Round 1, 30 breeding cages will be established, 240 mice per round.
Task 2	Month 3	Wean litters, MPA pellet implantation
Task 3	Months 4-6	MNU treatment of mice
Task 4	Month 7	MPA booster
Task 5	Months 4 - 8	Palpations of mammary glands for tumor formation and progression twice weekly, Caliper measurements of tumor sizes
Task 6	Month 9	Terminate mice, tumors taken for pathological analysis
Task 7	Months 10 - 12	Statistical Analysis of results and pathological analysis of tumors

Specific Aim 2: to examine the effect of immune modulation on the growth rate of tumors in Muc1-deficient and control mice.

Task 8	Month 4	Round 2 of mice produced for accurate determination of timing of tumor initiation (240 mice)
Task 9	Month 5	Wean litters, MPA implantation
Task 10	Month 6 - 8	MNU treatment of mice
Task 11	Month 9	MPA booster
Task 12	Month 6 - 9	Sacrifice mice at weekly intervals (5/group), mammary glands taken for pathological analysis

Specific aim 3: to examine the characteristics of mammary tumor cells derived from the Muc1 deficient, transgenic, and control mice to recognize and bind extracellular matrix ligands including fibronectin, laminin, collagen type iv and v, E-selectin, and hyaluronic acid.

Task 30	Month 8-33	Disaggregate tumor cells and measure binding to purified extracellular matrix components on microtiter plates

During August of the first year of this grant, mice were bred to yield sufficient numbers of breeders to produce approximately 75 females for each treatment group in the tumor growth study, specific aim one. In mid-October, 15 breeding cages of Muc1 homozygous mutant mice on the C57BL/6 background (Muc1 -/-; two females and one male per cage) were set up. Another 15 breeding cages with C57BL/6 wildtype (Muc1 +/+; two females and one male per cage) were set up. Trial pups were born in early November and were weaned approximately three weeks after birth. Females were separated five per cage at weaning. Individual females were identified by clipped toes. Animal identification consisted of a number comprising the genetic label (Muc1 +/+ = wt; Muc1 -/- = ko), cage number and toe number.

Treatment groups of Muc1 +/+ females (n=75) and Muc1 -/- females (n=75) surgically received 60-day time release implants of medroxyprogesterone (MPA; 40 mg/pellet) at two months of age. Mice were anesthetized with Avertin (2.5%; 0.3 ml/20 g mouse). Implants were inserted subcutaneously through a 2-3 cm incision above the spine between the scapula and the incision was closed with surgical staples.

A week following medroxyprogesterone implantation, the mice received an initial injection of nitroso-methylurea (NMU; 5 mg/100 g of body weight in isotonic 8.7 mM sodium phosphate buffer). The females at this age averaged 20 grams. Each animal received 1 mg NMU in 100 ul carrier. Injections were interperitonial. Subjects received a second NMU treatment one month later. A third NMU injection was given to survivors two months after the initial treatment. Remaining scheduled injections (number 4 and 5) were cancelled due to mortality among treatment group mice.

Inordinate and unsuspected mortality occurred early in the treatment regime. After the initial NMU injection, 43% of the Muc1-/- subjects died. Postmortem inspection revealed no morphological abnormalities such as hemorrhaging, enlarged liver, spleens or other organs. Mice died three to four days after injection. Mice rejected feed or water the day or two prior to death. Mortality among the Muc1-/- animals stabilized over the next two injections, while Muc1 +/+ individual losses were steady and at a slower rate. However, after the third injection of NMU in March, total animals remaining on trial were only 51% numbers initially starting the trial. Surviving animals appeared thin with dull coats. After six months, approximately a fifth of the original test animals remained alive. Overall health was deemed poor, and the trial was discontinued due to health failure of the mice after NMU treatment.

During the six months of the trial, only one mammary tumor was generated among wildtype mice. A left inguinal mammary gland tumor was first detected (approximately 1 cm) at 40 days post initial NMU injection. Within three weeks of detection, the fast growing tumor had developed to approximately 10% of its body weight. The animal was sacrificed when its health failed. The tumor was harvested, placed in methacarn (60% methanol, 30% chloroform, 10% acetic acid) for 2 hours, then stored in 75% ethanol until sectioning. Methacarn fixed tissues were embedded in paraffin blocks and sectioned to a thickness of 5 μm. Muc1 protein was

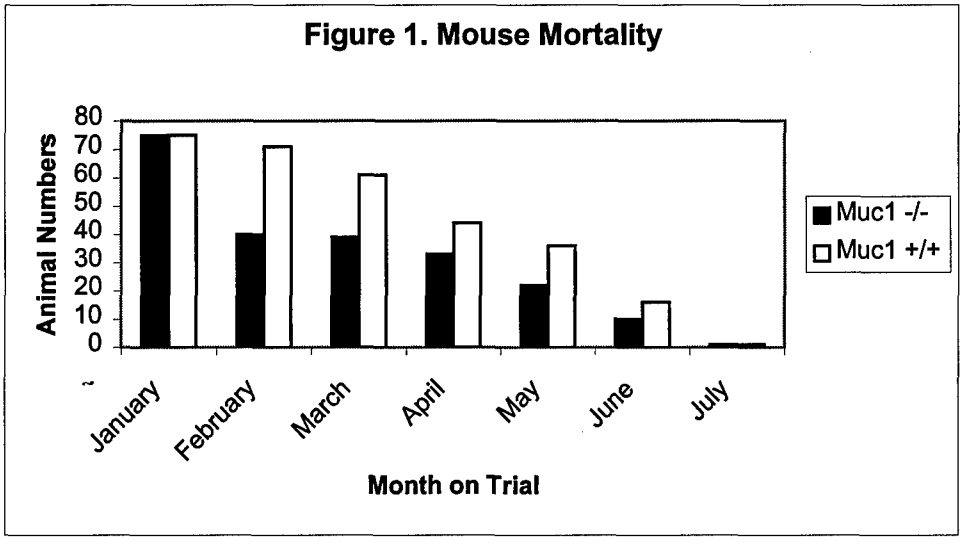


Figure 1. Mouse Mortality. Muc1 +/+ and Muc1 -/- mice were implanted with MPA time release pellets followed by monthly injections of NMU. The numbers of live animals declined precipitously for knockout Muc1-/- after the first injection. Both wildtype and knockout mortality remained steady throughout the six months before the trial was aborted.

detected using CT1, a polyclonal antiserum raised to a synthetic peptide in the cytoplasmic tail of MUC1 (Pemberton, et al..,1992). Control samples were incubated with the synthetic peptide sequence recognized by the α-MUC1 antibody generated in rabbit. Prior to staining, the slides were then blocked with 50% fetal calf serum in PBS for 1.5 hr at 25°C. The tissues were subsequently incubated at 25°C for 1.5 hr with an appropriate dilution of primary antiserum. After a series of 3 five minute washes in PBS, the samples were incubated at 25°C for 1.5 hr with a secondary antibody conjugated to Cyanin-3. The tissues were again washed as described above and coverslips were applied using Gel/Mount (Biomeda, Foster City, CA). Staining was observed and photographed with a Nikon Microphot - FXA. Positive labeling of mammary tumor tissue by CT1 indicated the presence of Muc1 in NMU/MPA induced tumors in mice.

The disappointing morbidity and lack of tumor induction due to treatment of mice with NMU/MPA derailed the agenda set forth in the Statement of Work for this proposal. While we recognized at the onset of the trial potential pitfalls and problems stemming from treatment with chemical carcinogens, the drastic effect of NMU on our murine models was beyond our worst case estimate and proved insurmountable. Lack of tumor development and mouse mortality made progress toward completion of Tasks 5-30 impossible. To compensate for the loss of tumor induction by chemical carcinogenesis proposed in this study, this laboratory revamped our genetic approach to inducing mammary tumors.

An intense breeding project was commenced during the spring to generate Muc1 mutant mice that were on a congenic FVB background. These Muc1 mutant or wildtype

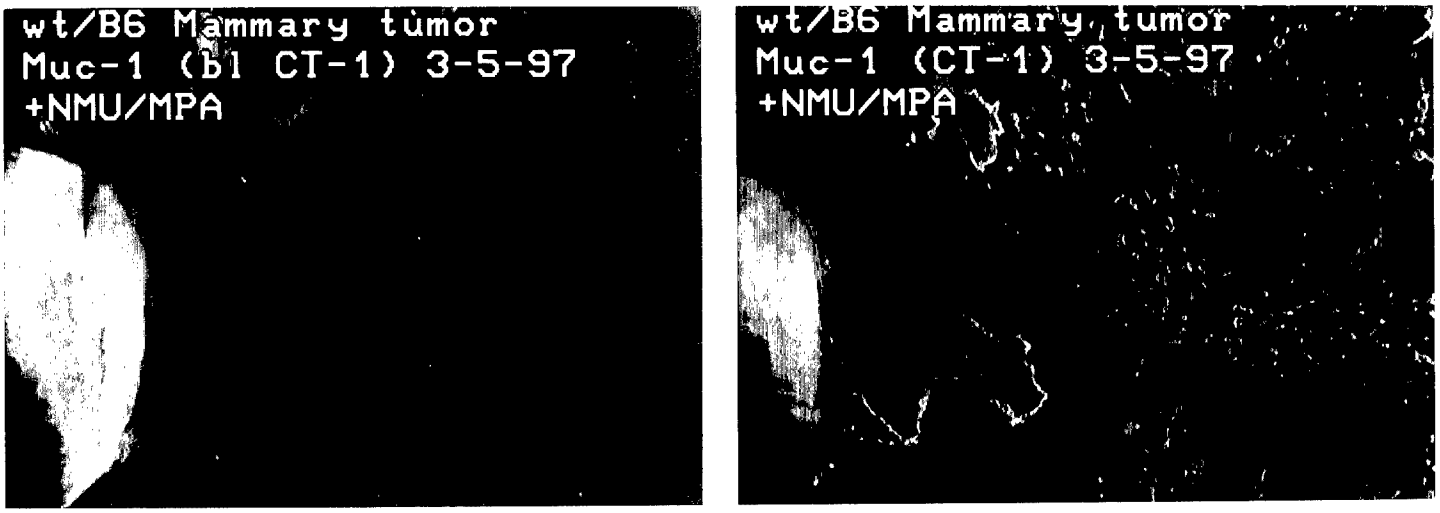


Figure 2. Muc1 expression in chemically induced mammary tumor tissue from mouse. a) Tissue incubated with polyclonal CT1, an antibody specific for the cytoplasmic tail region of human MUC1, blocked prior to incubation with a sythetic peptide representing the antigenic sequence; b) Tissue incubated with non-blocked CT1.

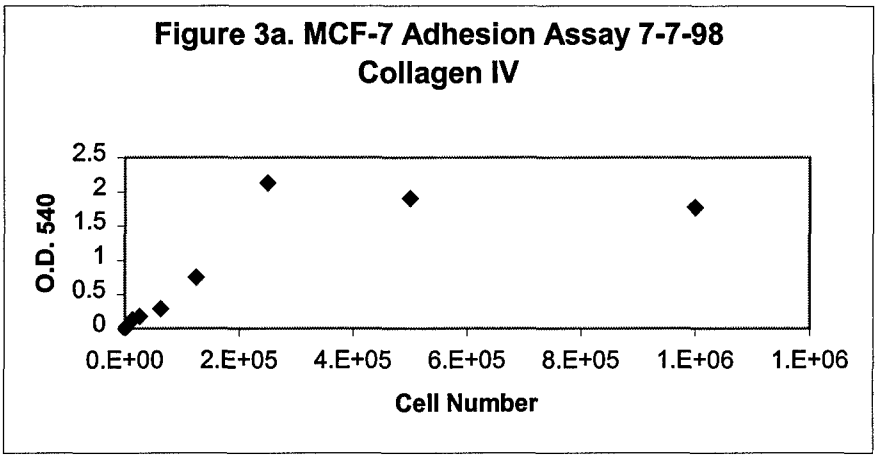
mice would then be mated with mice carrying the unactivated c-neu oncogene under transcriptional control of the mouse mammary tumor virus (MMTV). This transgenic model develops tumors in 50% of females by age 205 days, with incidence observed as early as 4 months of age. These tumors are Muc1 positive as determined by CT1 labeling. Mammary tumors arising in these transgenic mice proffer a suitable model for human breast adenocarcinomas overexpressing neu. Human primary breast cancers frequently overexpress neu (Slamon, 1987). Significant numbers of MMTV-neu transgenic Muc1 knockout and wildtype mice for tumor growth studies have been generated in our animal facility. Trial animals are currently three to four months of age, and tumor incidence and development is being screened by regularly scheduled palpation. Tumors generated from the unactivated neu model will allow progress toward completion of each specific aim outlined in the Statement of Work.

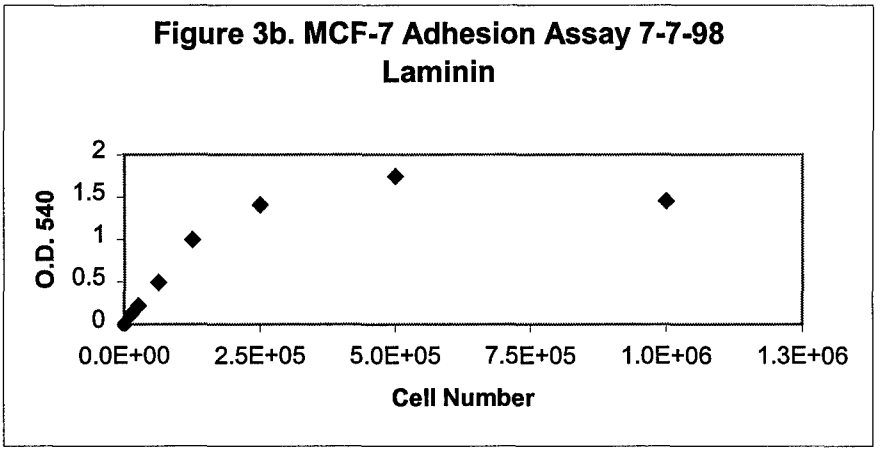
Using tumor tissue available from alternative murine breast cancer transgenic models and breast cancer cell lines, progress was made toward setting up assays for cell/matrix component adhesion (Task 30). Standard curves for estimate of cell number adherence to extracellular matrix components were constructed using the human MCF-7 cell line to establish and characterize the assay. MCF-7 cells were grown to approximately 50% confluence. Cells assayed for adherence were used within 1-2 days after passage. Cells were dissociated from culture plates with 0.2% EDTA in 1x PBS (Versene 1:5000; Gibco BRL, Grand Island, N.Y.) for 10 minutes at 37°C. Cells were rinsed 2x with PBS and pelleted in a centrifuge (2,000 rpm, 2 minutes). Cells were diluted serially ($1x10^6$ to $1.3x10^4$ cells/100 ul) in PBS containing $CaCl_2$ (0.1g/l) and $MgCl_2 \cdot 6H_20$ (0.1g/l) for plating on ECM coated 96 well plates. Assays were performed on laminin, collagen iv, and vitronectin coated 96-well plates (CytomatrixTM cell

adhesion strips, Chemicon International, Temecula, CA). Wells were rehydrated with 200 ul PBS for a minimum of 15 minutes at 25°C prior to application of serially diluted cells. Triplicate samples of each cell dilution in 100 ul were added to each well. Samples were incubated at 37°C for 1.5 hours in a $C0_2$ incubator. Free cells in suspension were carefully removed from wells, and the plates were gently submerged in assay buffer three times. After removal of all wash from the wells, samples were incubated in 0.2% crystal violet (100 ul; 0.2 g Crystal violet in PBS plus 10% ETOH) for five minutes. Crystal violet was removed and wells were submerged in assay buffer three times. Lysis buffer (100 ul; 1% SDS in PBS plus 50% ETOH) was added to each well, and plates were incubated on a shaker platform at 25°C until cells were lysed, approximately 5 minutes. Absorbency of lysate was determined at 540 nm. Standard curves for MCF-7 cell adherence to laminin, vitronectin and collagen IV were linear up to approximately $2.5x10^5$ cells/100ul.

To determine whether murine tumor cells adhered to vitronectin, tumor cells were disaggregated under mild conditions and incubated on ECM coated plates. Tumors were obtained from transgenic mice carrying the unactivated neu oncogene driven by the middle T-antigen (MTag) on the C57BL/6 background or 129 background. Tumors were surgically removed, and tissue was minced with a scalpel in 0.5% EDTA in PBS (1X). Tissue was incubated for 15-20 minutes at 37°C on a shaker platform. Tissue was then forced through a 40 um nylon cell strainer (Falcon, New Jersey). Cells were collected, washed 3x in PBS containing $CaCl_2$ (0.1g/l) and $MgCl_2 \cdot 6H_20$ (0.1g/l), and suspended at a concentration of approximately 1 x 10^6 cells/100 ul. Dissagregated tumor cells densely adhered to vitronectin (Figure 4a). Assays performed in parallel to the tumor cell adhesion assays indicated that MCF-7 cells (Figure 4b) and the murine cell line C57 (Figure 4c) also bound to vitronectin coated plates. Controls in these assays included BSA coated wells. Adherence to control wells by each type of cell was null.

Results from these preliminary cell adhesion assays indicate that both murine tumor cells and murine mammary tumor cell lines adhere to ECM components coating 96 well plates. Assays describing interactions between Muc1 positive and Muc1 negative





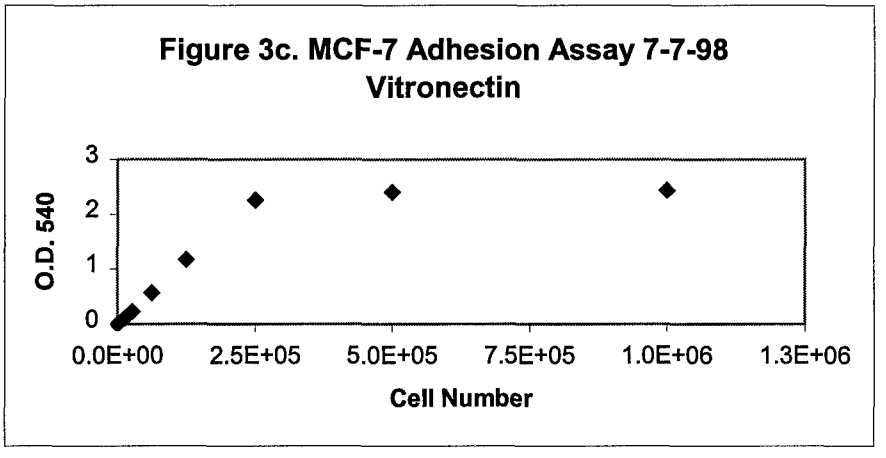
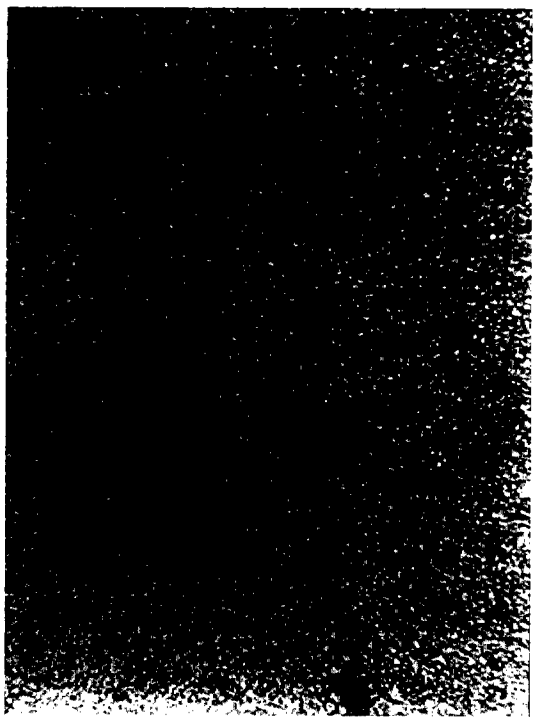


Figure 3. MCF-7 cell adherence standard curve to ECM components. MCF-7 cells adhering to a) collagen IV; b) laminin; c) vitronectin.

Figure 4. Interaction of human and murine cell lines and murine tumor cells with ECM. a) Disaggregated murine Muc1 knockout tumor cells on vitronectin. b) Murine Muc1 knockout tumor cells after careful washing and staining with crystal violet. c) MCF-7 human carcinoma cells bound to vitronectin. d.) Murine cell line C57 bound to vitronectin

4a



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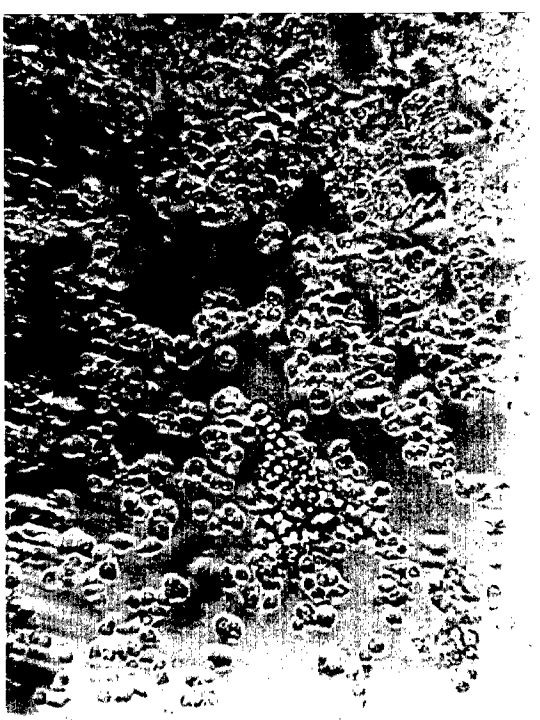

4d

mammary tumor cells may contribute to the understanding of adherence characteristics innate to cells expressing or lacking Muc1.

Conclusions

Chemical carcinogenesis using NMU for the induction of mammary tumors is well documented in rats. Murine response to NMU is only briefly documented in the literature (Pazos et al., 1991). Following the protocol for NMU induction of mammary tumors delineated in the Pazos paper, this laboratory experienced excessive mortality in our mouse models. While a tumor induced by this method did express Muc1, no comparisons could be made in tumor growth, immune response, or cell/matrix components due to the lack of tumors and the morbidity among trial animals. NMU/MPA chemical carcinogenesis does not induce mammary tumors in our C57BL/6 based mammary tumor models.

The objectives set forth in this proposal can be addressed by alternative models in which tumor incidence and growth are driven by genetic means rather than by the administration of chemicals. We have developed virtually purebred (>99%) mice that have been genetically modified to include the unactivated neu oncogene driven by the murine mammary tumor virus (MMTV) promoter. These mice are on the FVB background and include either the wildtype Muc1 gene (Muc1 +/+) that express the mucin, or the mutant knockout Muc1 gene (Muc1-/-) which are null for Muc1 expression. These mice provide a viable, efficient opportunity to examine the effects of Muc1 expression on tumor growth, immune response to tumors, and the ability of tumor cells to interact with extracellular matrix components in models that represent human tumor characteristics. This study is in progress. Presently 65 mice have been enrolled on the c-neu-Muc1+/+ arm and 35 mice on the c-neu-Muc1-/- arm. Mice are being palpated every week and additional mice are being bred for the c-neu-Muc1-/- arm.

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